

## **REMARKS/ARGUMENTS**

Please cancel claims 2-5, 9-10 and 24-26, without prejudice or disclaimer, and amend claims 1, 21 and 23 as indicated. As a result of the cancellation and amendment of these claims, the claims currently under consideration are directed to a region on chromosome arm 4p. Applicants reserve the right to prosecute in a further application the canceled claims, which are directed to chromosomal regions other than the region in chromosome arm 4p. Claims 1, 6-8, and 11-23 are pending. Claims 13, 14 and 27 have been withdrawn.

Applicants assume that the amendment to the first sentence of the specification, which properly claims priority to the relevant patent applications, thereby obviating the objection by the Examiner in the Office Action of October 3, 2004, was entered by the Examiner following its presentation in the Reply filed March 3, 2004.

Applicants gratefully thank Examiner Fredman for his courtesy in holding an informal telephonic interview with the undersigned concerning the Advisory Action. The amendments and arguments in the present Response reflect the substance of that interview and, it is believed, place the application in condition for allowance.

### **Rejections under 35 USC §112**

The claims currently under consideration are directed to a region within the human genome that is associated with resistance to (protection from) familial bipolar affective disorder (BPAD). Markers in this region, which is located on chromosome arm 4p, are shown in Table 7 and Figure 6 of the specification. The inventors have identified about 25 markers in this region which exhibit a highly significant correlation to resistance to BPAD (have a SIBPAL p value of  $<0.05$ ). About 19 of those markers show a SIBPAL value of  $<0.01$ , and many of those show a SIBPAL value of  $<0.001$ . This region represents a relatively limited region in which one can, e.g., screen members of a family for these or for further markers that are correlated with BPAD. (The broader region, between *D4S431* and *D4S404*, which is recited, e.g., in claim 1, extends for about 33 cM. The narrower region, between *D4S30071* and *D4S19*, which is recited, e.g., in claim 6, extends for about 20 cM.) Within this region, one can determine one or more markers (a genotype) that are linked, in a particular family of interest, to protection from BPAD. Alternatively, the claimed region

provides the basis for narrowing the size of the region that is linked to BPAD, or for strengthening the statistical correlations shown in this application. The above types of analyses can be accomplished by screening additional markers; by using the disclosed markers, *e.g.*, the markers recited in claims 7 and 8; and/or by studying larger and/or different cohorts of subjects. The methods of the invention allow the formulation of sufficient detail such that determination of an allele(s) associated with increased resistance to BPAD may be determined (see, *e.g.*, page 5, lines 25-28 of the specification).

Thus, the instant claims are directed to screening methods, *e.g.*, a method for determining a genotype associated with increased resistance to (protection from) familial bipolar affective disorder (BPAD) ... (independent claim 1); or a method for determining the contribution of a chromosomal region to the presence of resistance to BPAD... (independent claim 23). In the method of claim 1, for example, a genotype of a (at least one) family member is determined; the BPAD status of the family member is determined; the genotype is compared with the BPAD status; and from this comparison, it is determined if the genotype is associated with increased resistance to BPAD.

The enablement and the written description rejections in the Office Action of October 3, 2004 and repeated in the Advisory Action of March 31, 2004 appear to be directed to claims that recite a genotype which is associated with increased or decreased resistance to bipolar disorder (*e.g.*, the *result* of a screening method for determining a genotype associated with increased or decreased resistance to BPAD). This is not what the instant claims recite. As discussed in the Replies filed on July 22, 2003 and March 3, 2004, and again below, the specification provides both enablement and written description for performing the claimed screening methods.

With regard to the identification of the presently claimed region, the Reply of July 22, 2003 reviewed in detail that at least one marker within the claimed region is significantly correlated with protection from BPAD. That is, the marker *D4S2949* on chromosome arm 4p exhibits statistically significant linkage to resistance to (protection from) BPAD, with a SIBPAL p value of 0.0000, or  $< 5 \times 10^{-5}$  (see, *e.g.*, Figure 6 and page 44, lines 11-13). The Examiner has presented no reasons or evidence to doubt the assertion that this marker is highly correlated with resistance to BPAD. In fact, the Examiner acknowledges that this linkage is significant in paragraph 5 of the Advisory Action of March 31, 2004.

The demonstration of this strongly correlated marker would, in itself, be sufficient to identify this region for further genotypic screening. It is well-known to those of skill in the

art that one would reasonably expect that markers located at about 10cM (or, in some cases, more) from a marker of interest would be linked to that marker. Applicants submitted an article with reference in the Reply of March 3, 2004 showing that this distance is well-established to contain linked markers. However, in paragraph 5 of the Advisory Action, the Examiner objected that the reference was published after the effective filing date of this application, and thus allegedly did not establish enablement. We now attach an article published in 1995, which is well before the effective filing date (March 29, 1996) of the application. This article - Brink *et al.* (1995) *Circulation* 91, 1633-1640 - shows linkage analysis which determines the approximate chromosomal position of a gene causing Progressive familial heart block type I (PF-HBI). The *PFHBI* locus was mapped to an area of approximately 10 cM on a particular chromosome.

The present application goes beyond this initial identification of a region of interest, by demonstrating numerous additional markers within this claimed region that exhibit a strong correlation to resistance to BPAD. These data are shown in Figure 6 and in Table 7 in the specification. The Figure presents selected data points from the larger number of data points presented in the Table. A summary of data identifying some of these additional markers is presented in Appendix A. In response to the request by the Examiner on page 4 of the Office Action of October 3, 2004, the Table in which these data appear is explained in detail in the Appendix. Briefly, on chromosome arm 4p, at least 19 markers have been identified within the 33.3cM region between *D4S431* and *D4S404* which are correlated with resistance to bipolar disease in a statistically significant manner.

In the methods recited in the instant claims, members of a family of interest are tested for the presence of markers that lie within the indicated region. As noted above, it would be evident to a skilled worker that markers within about 10 cM of a highly correlated marker identified in the application are likely to be linked to that marker, thereby defining a linkage region of interest. In fact the currently claimed region of interest is even more well characterized. For chromosome arm 4p, at least about 19 markers with statistical significance are spread across about 33cM; thus, the region of interest extends for at least this length.

The identification of this region of interest is supported throughout the specification. The specification is replete with sophisticated analyses of the data, employing a variety of statistical techniques, which support the significance of the linkage. The fact that most of the data presented in the application have been published in prestigious peer review journals confirms the validity of these conclusions. Two such papers were attached to the Reply of

March 3, 2004 for the convenience of the Examiner: Ginns *et al.* (1996) *Nature Genetics* 12, 431-435; and Ginns *et al.* (1998) *Proc Natl Acad Sci U S A* 95, 15531-15536. The allegation in the Office Action that other studies of linkage in BPAD may have been faulty (*e.g.*, as reported in the Berrettini reference cited by the Examiner, and as acknowledged in the specification, for example at page 2, line 27 to page 3, line 2) does not cast doubt on the accuracy or significance of the present findings.

**The fact that several markers within this region of interest may not appear to be statistically significant does not cast doubt on the conclusion that the region is correlated with resistance to (protection from) BAPD. Only three markers of the approximately 28 markers tested from this region - D4S2935, *Afm087zg5*, and D4S2984 - show a SIBPAL p value of >0.05. The inventor has assured us that these three markers, which lie within a region identified as showing significant linkage, but which by themselves do not exhibit such linkage, almost certainly are non-informative. A "non-informative marker" is one for which there is no variation among members of a population (in the present case, the Old Amish population which was studied). Therefore, the presence or absence of such a non-informative marker in a subject in the present studies has no bearing on whether the individual is sensitive to or resistant to bipolar disease.**

**To reiterate, about 28 of the 31 markers tested from the claimed region of chromosomal arm 4p exhibited significant linkage to resistance to (protection from) BAPD. Clearly, a skilled worker would have a reasonable expectation that, without undue experimentation, s/he could identify a marker from the claimed region which would exhibit such linkage.**

The Examiner appears to be concerned that the Blackwood reference (*Nature Genetics* 12, 427-430, 1996) cited in the Office Action identifies some markers in chromosome arm 4p as being associated with susceptibility to BPAD, whereas the present inventors identify markers in this region as being protective for BAPD. This is not a problem. It is not uncommon to find alleles within a given chromosome region, or even mutations within a given gene, that result in opposite phenotypic effects. The existence of such apparently contradictory results has been confirmed by several post-filing studies. For example, as reported in the Geller *et al.* reference attached to the Reply of March 3, 2004, some mutations in the melanocortin-4 receptor gene predispose a subject to obesity, whereas other alleles in this gene have a negative association with obesity. Furthermore, three other articles attached

to that Reply (Sevush *et al.* (2000); Beyer *et al.* (2002); and Higgins *et al.* (1997)) showed that certain isoforms of ApoE (*e.g.*, ApoE 2) are protective for Alzheimer's Disease, whereas others (*e.g.*, ApoE 4) are associated with susceptibility to that disease. The present inventors have recognized that markers on chromosome arm 4p correlate with *protection* from BPAD, an association that was not reported by Blackwood.

As for the allegation in the Office Action of October 3, 2004 on page 7 that “The ability to screen for a wellness allele is even more unpredictable because it is very difficult to distinguish between the presence of a protective allele and the absence of a susceptibility allele,” the specification clearly teaches how to distinguish between these two possibilities.

For example, the specification states at page 39, lines 9-16 that

Importantly, because of the long-term, longitudinal nature of the study, even the unaffected, mentally healthy individuals (those without any psychiatric illness) in these families have been closely followed, many for a period of years past the age of risk for BPAD. Consequently, rather than limit this genome-wide search to identifying susceptibility loci for the disease phenotype (BPAD), we tested the hypothesis that “protective” alleles may contribute to the absence of psychiatric illness (*i.e.*, mental health ‘wellness’) in unaffected family members in the ‘high risk’ pedigrees.

and at page 51, lines 13-16:

Accordingly, an important step in our study which demonstrates that there are “protective” alleles was to show that there are “mentally healthy” individuals who share marker alleles that should increase the risk of developing BPAD, and yet, in the presence of ‘protective’ alleles these individuals do not manifest BPAD.

The existence of markers associated with wellness is well-established in the art. See, *e.g.*, the specification at page 54, line 16 to page 55, line 4. For example, as discussed in the Reply filed March 3, 2004, wellness alleles have been reported for viral infections and Alzheimer's disease. Again, the fact that the well respected journal, *Proc. Natl. Acad. Sci, USA*, published the above-mentioned paper by the present inventors, which shows that the markers on chromosome 4p from the present application are linked to a protective effect for BPAD, attests to the convincing nature of those data.

The specification is fully enabling for the recited screening methods: it teaches how to obtain samples from family members (*e.g.*, at page 19, lines 12-27 and Example I); how to perform the genotypic analysis (*e.g.*, in Example II); how to assess BPAD status (*e.g.*, in Examples I and IV) and how to analyze statistically whether the genotype determined is

associated with increased or decreased resistance for BPAD (*e.g.*, in Example III). The specification teaches a variety of markers that can be used in the claimed methods, and teaches how additional markers can be generated (*e.g.*, at page 23, line 27 to page 27, line 10). The Office Action of October 3, 2004 states at page 7 that “While one could conduct additional experimentation to determine whether markers exist within the recited regions on chromosome 4 and 11 and these newly discovered markers are associated with BPAD, the outcome of such research cannot be predicted.” That is to some extent correct. The claimed methods are screening methods, directed toward determining whether markers in the recited region of chromosomal arm 4p are, in fact, associated with BPAD. **As discussed above, about 25 of the 28 markers in this region which were tested showed significant correlation with resistance to BAPD. Clearly, it would not require undue experimentation to identify additional correlated markers by screening markers from this region.**

Not only is the specification enabling for the claimed invention, but it also provides written description. Contrary to the allegation of the Examiner on pages 12-14 of the Office Action of October 3, 2004, and repeated in the Advisory Action of March 31, 2004, the specification provides even more than a “representative number” of markers within the claimed region of the chromosomes. As discussed above, only one highly correlated marker is required to identify a region for further screening. In fact, the present application provides many more markers (about 25) within the presently claimed region. Some suitable markers to test for resistance to BPAD, flanking *D4S2949* (on chromosome arm 4p), are indicated at page 14, line 31 to page 15, line 4. See also further candidate markers for resistance indicated on page 18, lines 5-13. Examples of suitable primers for identifying the above markers in chromosomal arm 4p are indicated on page 22, line 1 to line 19.

In view of the preceding amendments and arguments, the application is believed to be in condition for allowance, which action is respectfully requested. Should any issues remain that may be resolved by a telephone discussion, the Examiner is invited to telephone the undersigned.

Should any additional fee be deemed due, please charge such fee to our Deposit Account No. 22-0261 and advise us accordingly.

Respectfully submitted,

Date: June 3, 2004



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## APPENDIX A

Table 7 (pages 46-47; region on chromosome arm 4p) presents results of some of the linkage analyses presented in the above-referenced application. The Table presents SIBPAL analysis of 4p markers, in either a single pedigree (110) or in a total of four pedigrees (110, 210, 310, 410). Nominal and simulated values are presented for each marker.

Some of the significant linkages are summarized below:

On **chromosome arm 4p**, at least 19 markers have been identified within the ~33.3cM region between *D4S431* and *D4S404* that are correlated with bipolar disease in a statistically significant manner. These data are shown in Table 7. In a few cases, significant values for markers are also shown Fig. 6:

<u>Marker</u>	<u>Fig. 6 (SIBPAL p)</u>	<u>Table 7 (SIBPAL p, nominal, pedigrees 110-410)</u>
D4S431		0.0110 (pedigrees 110-410)
D4S2366	0.0002	0.0002
D4S3007	0.0015	0.0014
D4S394	0.0035	0.0007
D4S2983		$<1 \times 10^{-4}$
D4S2923		0.0003
D4S615		$<1 \times 10^{-4}$
Afma184xa9		$<1 \times 10^{-4}$
D4S2928		$<5 \times 10^{-5}$
D4S1582		0.0032
D4S107		$<5 \times 10^{-5}$
D4S3009		0.0001
D4S2906		0.0004
D4S2949	0.0000	$<1 \times 10^{-7}$
D4S1582	0.0005	
D4S2942		$<1 \times 10^{-4}$
D4S3048		0.0036
D4S419	0.0002	0.0004
D4S404	0.0004	0.0004

Also, D4S391, which lies outside of the claimed region, is shown in Table 7 to have a SIBPAL  $p=0.0001$  (pedigree 110).

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## Articles

### Gene for Progressive Familial Heart Block Type I Maps to Chromosome 19q13

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## ▶ Abstract

*Background* Progressive familial heart block type I (PF-HBI) is a dominantly inherited cardiac bundle-branch conduction disorder that has been traced through nine generations of a large South African kindred. Similar conduction disorders have been reported elsewhere; however, the cause of these diseases is unknown. The aim of the present study was to determine by linkage analysis the approximate chromosomal position of the gene causing PFHBI, thereby allowing family-based diagnosis and the development of positional cloning strategies to identify the causative gene.

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*Methods and Results* Eighty-six members of three pedigrees, 39 members of which were affected with PFHBI, were genotyped at four linked polymorphic marker loci mapped to chromosome 19, bands q13.2-q13.3 (chromosome 19q13.2-13.3). Maximum two-point logarithm of the odds scores (which represent the logarithm of the odds ratio of detecting linkage versus nonlinkage) generated were 6.49 ( $\Theta=0$ ) for the kallikrein locus, 5.72 ( $\Theta=0.01$ ) for the myotonic dystrophy locus, 3.44 ( $\Theta=0$ ) for the creatine kinase muscle-type locus and 4.51 ( $\Theta=0.10$ ) for the apolipoprotein C2 locus. The maximum multipoint logarithm of the odds score was 11.6, with the 90% support interval positioning the *PFHBI* locus within a 10 cM distance centering on the kallikrein 1 locus.

*Conclusions* The gene for PFHBI maps to an area of approximately 10 cM on chromosome 19q13.2-13.3. There are several candidate genes in this interval; although a recombination event ruled out the myotonic dystrophy locus from direct involvement with PFHBI, the proximity of these two loci may be relevant to the observed cardiac abnormalities of myotonic dystrophy. The results provide a means of DNA-based diagnosis in the families studied and a foundation for cloning studies to identify the causative gene.

**Key Words:** conduction • bundle-branch block • mapping • genes

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## ► Introduction

Progressive familial heart block type I (PFHBI) is an autosomal dominantly inherited

cardiac bundle-branch disorder that may progress to complete heart block.<sup>1 2 3 4</sup> It is defined on ECG by evidence of bundle-branch disease, ie, right bundle-branch block, left anterior or posterior hemiblock, or complete heart block with broad QRS complexes.<sup>1</sup> Progression has been shown from a normal ECG to right bundle-branch block and from the latter to complete heart block.<sup>2 3</sup> These ECG features differentiate PFHBI from progressive familial heart block type II (PFHBII), in which the onset of complete heart block is associated with narrow complexes.<sup>1</sup> Electrocardiographically the changes represent, respectively, bundle-branch disease (PFHBI) and atrioventricular nodal disease with an atrioventricular block and an idionodal escape rhythm (PFHBII). PFHBI is manifested symptomatically when complete heart block supervenes, either with dyspnea, syncopal episodes, or sudden death.<sup>1</sup> Treatment, which is best managed by regular ECG follow-up, is by the timely implantation of a pacemaker.

Several branches of a large kindred in South Africa have been identified in which PFHBI is segregating and whose family members descend from one ancestor who emigrated from Portugal in 1696.<sup>1</sup> It has been estimated that there may be between 1000 and 9000 gene carriers among the descendants.<sup>1 5 6</sup> Although the global incidence of PFHBI is unclear, the disease is probably not confined to South Africa. At least 13 reports suggest that similar familial conduction diseases, ie, with right bundle-branch block or right bundle-branch block and complete heart block appearing in the same family, exist elsewhere, although designated differently.<sup>10</sup>

The pathophysiology of these diseases is unknown; however, linkage analysis and positional cloning<sup>7 8 9</sup> offer a means of identification of disease-causing genes. Characterization of genes that predispose individuals to the development of electrophysiological disturbances may help elucidate the functioning at the molecular level of the cardiac conduction system, whether normal or abnormal.

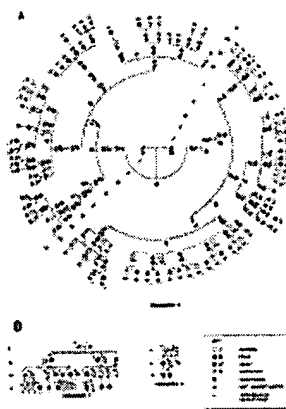
We have previously reported exclusion of 68 loci, representing 35% of the genome, from linkage to PFHBI.<sup>10</sup> In this paper we report linkage of PFHBI to chromosome 19, bands q13.2-q13.3 (chromosome 19q13.2-13.3). The gene encoding myotonin protein kinase (*DMK*), which is implicated as a cause of myotonic dystrophy, lies within this region.<sup>11 12</sup> <sup>13</sup> Myotonic dystrophy is a disease that is itself complicated by heart block and other conduction abnormalities.

## ► **Methods**

## Subjects and Clinical Evaluation

Subjects were drawn from three pedigrees identified through previous studies.<sup>1 5 6</sup> A consolidated pedigree of one large South African kindred, pedigree 2, which could be traced back nine generations, and in which PFHBI segregates, is shown in Fig 1A+. These data incorporate the previously reported pedigrees 2, 3, 4, and 8<sup>10</sup> and other family members who have since come to our attention. The previously reported pedigrees 1 and 5<sup>10</sup> (Fig 1B+) have not as yet been linked to the larger pedigree. However, because most members of all three pedigrees live in or originate from the same geographical area, the Eastern Cape in South Africa, and given the rarity of the disease, we expect a common ancestor.

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**Figure 1.** Diagrams of pedigrees in which PFHBI segregates. A, Pedigree 2, a nine-generation kindred. B, Pedigrees 1 and 5, two smaller families that could not be linked to the main pedigree. Affected individuals had either right bundle-branch block, complicated right bundle-branch block, or complete heart block, as described in the text.

The diagnosis of PFHBI followed strict criteria, based on ECG-defined right bundle-branch block, complicated right bundle-branch block, or complete heart block with broad complexes in the absence of disease that might cause a similar defect, as previously described.<sup>10</sup> Sinus bradycardia in isolation was regarded as normal. The Minnesota code was adhered to in making ECG diagnoses.<sup>14</sup> Whenever available, members of the pedigrees were entered into the study irrespective of age.

## DNA Analysis

Genomic DNA was extracted from lymphocytes or Epstein-Barr virus-transformed cell lines as previously described.<sup>15</sup> To retrospectively obtain a repeat sample from a patient who died during the period of study, we extracted DNA from archival postmortem tissue sections using a method modified from Shibata et al.<sup>16</sup> Individual sections 5 to 10  $\mu$ m thick were cut from trimmed, buffered, formalin-fixed, paraffin-embedded tissue and

placed in 500- $\mu$ L Eppendorf tubes. The samples were deparaffinized by extraction with 1 mL of xylene followed by microcentrifugation. This extraction procedure was repeated and 1 mL of 95% ethanol was added to the residue. After microcentrifugation the ethanol wash was repeated, and the pellet was desiccated before suspension in 40  $\mu$ L water. The solution was boiled for 10 minutes and microcentrifuged for 5 minutes, and 3  $\mu$ L of supernatant was used to confirm polymerase chain reaction–based detection of the trinucleotide repeat at the myotonic dystrophy (*DM*) locus, as detailed below. Genotyping was performed at the apolipoprotein C2 (*APOC2*) locus,<sup>17</sup> the creatine kinase muscle-type (*CKMM*) locus,<sup>18</sup> the *DM* locus,<sup>11 12 13</sup> and the kallikrein 1 (*KLK1*) locus,<sup>19</sup> which span chromosome 19q13.2-13.3. The microsatellite dinucleotide repeat polymorphisms at the *APOC2* and *KLK1* loci and the CTG trinucleotide repeat at the *DM* locus were analyzed by polymerase chain reaction–based assays. Primers were synthesized from published sequences (DNA Synthesis Laboratory, University of Cape Town, South Africa) for the *APOC2*<sup>17</sup> and *DM*<sup>11</sup> loci or purchased from Research Genetics for the *KLK1*<sup>19</sup> locus. Each reaction was performed in a 10- $\mu$ L volume that contained 300 ng of genomic DNA; 40 pmol of each primer; 67  $\mu$ mol/L each of dATP, dGTP, and dTTP; 2.5  $\mu$ mol/L of dCTP; 1.5 nCi of [ $\alpha$ -<sup>32</sup>P]-dCTP; 2.5  $\mu$ mol/L of MgCl<sub>2</sub>; and 0.75 U of *Taq* polymerase in the buffer supplied by the manufacturer (Promega). The reaction mixes were overlaid with 25  $\mu$ L of mineral oil to prevent evaporation. Cycling parameters in a polymerase chain reaction machine (ESU Electronics) were an initial incubation at 93°C for 2 minutes followed by 30 cycles at 93°C for 110 seconds, 57°C for 120 seconds, and 72°C for 140 seconds. After the addition of 6  $\mu$ L of loading dye (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanole, 20 mmol/L EDTA), 6  $\mu$ L of the amplification products were electrophoresed on 6% denaturing polyacrylamide gels. The gels were fixed and dried and the autoradiographs were exposed overnight at -70°C. The sizes of the different alleles were determined by reference to a sequencing ladder.

At the *CKMM* locus the *Nco*I and *Taq*I restriction length polymorphisms<sup>18</sup> were detected by Southern blot hybridization using methods previously described<sup>20</sup> or by a polymerase chain reaction–based method using published primer sequences.<sup>21</sup> The *Eco*R1 polymorphism at the *DM* locus was examined for possible expansion of the CTG repeat to pathological levels by Southern blot hybridization.<sup>22</sup>

### Statistical Analysis

MLINK (two-point) and LINKMAP (multipoint) from the LINKAGE group of programs were used to calculate LOD (which represent the logarithm of the odds ratio of detecting linkage versus nonlinkage) scores<sup>23</sup> from marker, disease status, and pedigree information using the following parameters. To obtain the penetrance value, input for the LIPED<sup>24</sup> program was constructed to reflect apparent penetrance and used in an iterative fashion to find the penetrance value that maximized the likelihood,<sup>25</sup> as previously described.<sup>10</sup> A maximal value was obtained at a penetrance of 96%. A more conservative value of 90% was used in the actual linkage calculations. Because an affection ratio of 0.45 or more was obtained upon examination of the number of affected individuals born in each decade since 1900, we concluded that assuming 90% penetrance would not influence linkage results negatively. The same phenotypic parameters were used for possible PFHBI homozygotes as for heterozygotes. The assumptions on which estimates of the prevalence

of PFHBI (0.002) were based were reported previously.<sup>10</sup> No allowance was made for phenocopies because the prevalence of similar cardiac conduction disturbances reported in the general population is low.<sup>10</sup>

To perform the multipoint analysis for the large multigeneration families with multiallelic markers, we used the following simplifying measures. In pedigree 2, all individuals in the line of descent of the PFHBI allele were coded as affected, with the exception of individuals I.01 and I.02, who were designated as being of unknown phenotype.<sup>6</sup> All individuals who married into the family were coded as unaffected. These modifications resulted in LOD score changes only after the second decimal digit when tested on a two-point analysis rerun. Alleles were then collapsed into three-allele systems for the *DM* and *KLK1* loci and a four-allele system for the *APOC2* locus. The individual alleles obviously segregating with PFHBI were maintained as separate alleles at the *DM* and *KLK1* loci. Only the *TaqI* restriction length polymorphism (ie, a biallelic polymorphism) was used at the *CKMM* locus. Allele frequencies were adjusted to incorporate these modifications and the simplified data were analyzed on a VAX 6000-410 computer system using published gene order and genetic distances.<sup>19 26 27 28</sup>

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## ► Results

### Clinical Evaluation

Table 1\* displays the clinical characteristics of subjects with PFHBI who were genotyped and in the additional affected first-degree relatives who were unavailable for genotyping. Of a total of 51 family members assessed, 22 had complete heart block, 25 had right bundle-branch block or right bundle-branch block and left posterior hemiblock or left anterior hemiblock, and 4 (individuals VIII.26 and VII.44 in pedigree 2 and I.01 and I.02 in pedigree 5) displayed no ECG abnormalities but had affected children.

View this **Table 1. Clinical Characteristics of Subjects With Progressive Familial Heart Block Type I Genotyped in the Study and Their**  
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Affected First-Degree Relatives

### Linkage of PFHBI to Chromosome 19q13

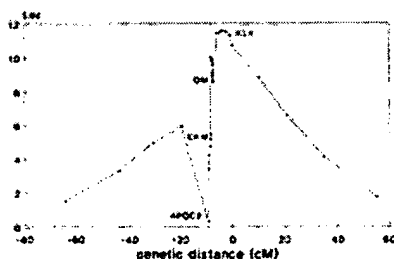
Eighty-six members of three pedigrees, 39 members of which were affected with PFHBI, were genotyped at four linked polymorphic marker loci mapped to chromosome 19q13.2-13.3. Results of pairwise linkage analysis between PFHBI and marker loci are summarized in Table 2+. The highest LOD score was obtained for *KLK1* ( $Z_{\max}$ , 6.49 at  $\Theta=0$ ). Marker locus *APOC2* ( $Z_{\max}$ , 4.51 at  $\Theta=0.10$ ) showed several recombinants, while in separate individuals *CKMM* ( $Z_{\max}$ , 3.44 at  $\Theta=0.05$ ) and *DM* ( $Z_{\max}$ , 5.72 at  $\Theta=0.01$ ) each showed a single recombinant. These genes span a distance of about 12 cM in the following order: centromere-*APOC2*-*CKMM*-*DM*-*KLK1*-telomere.<sup>26</sup>

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**Table 2.** Two-Point Logarithm of the Odds Scores of Progressive Familial Heart Block Type I and Chromosome 19 Markers by Pedigree

Marker	$Z_{\max}$	$\Theta$
<i>KLK1</i>	6.49	0.00
<i>DM</i>	5.72	0.01
<i>CKMM</i>	3.44	0.05
<i>APOC2</i>	4.51	0.10

The maximum LOD score for a multipoint analysis occurred at *KLK1* (LOD 11.6) (Fig 2+). The 1-LOD-down 90% support interval<sup>29</sup> defined an area of 10 cM centering on the *KLK1* locus as being likely to contain the PFHBI gene. This distance may include the *DM* locus.

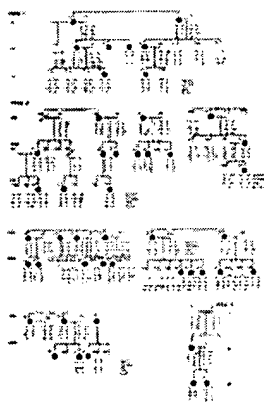


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**Figure 2.** LINKMAP plot of chromosome 19, band q13, showing the relative likelihoods for the various locations of the PFHBI gene with reference to a fixed order of linked DNA markers. On the basis of the 1-LOD-down support interval, the most likely location for the locus for *PFHBI* is distal to the *CKMM* locus, encompassing a 10 cM length centering on the *KLK1* locus.

### Recombination Event at the *DM* Locus

At the *DM* locus, one recombination event was seen between individual VIII.46 and IX.25 in pedigree 2 (Fig 3+). The latter, who was affected with PFHBI, inherited from his affected father the same length CTG repeat (allele 14) and *Eco*RI polymorphism (allele 2) at the *DM* locus as his unaffected sibling IX.24, who showed no evidence of recombination events. Because the *DMK* gene was a candidate for the cause of PFHBI, it was important to confirm the recombination event in IX.25. Unfortunately, this child had died at the age of 2 years, so a new blood sample was unobtainable. However, genomic DNA was extracted from formalin-fixed, paraffin-embedded postmortem material. The CTG repeat at the *DM* locus was polymerase chain reaction-amplified from this source and a genotype of 13:14 was confirmed. The mother (VIII.45) was not affected with PFHBI, as assessed by ECG testing, and to date no ancestral links between her and the PFHBI families have been found. Eighteen of the 37 PFHBI-affected individuals were heterozygous for alleles within the normal range of the myotonic dystrophy-causing trinucleotide repeat. All possessed a 5-repeat-length allele and one other allele of up to 21 repeats. One subject, IX.25, in whom the recombination event was seen, was heterozygous with alleles of 13- and 14-repeat lengths. The remaining affected individuals were apparently homozygous for the 5-repeat-length allele, a result compatible with their parents' genotype. Furthermore, in none of the affected individuals with the single 5-repeat-length band was there evidence of triplet repeat expansion, which might have been undetectable by polymerase chain reaction-based amplification, upon Southern blot analysis of the *DM* locus (results not shown).



**Figure 3.** Genotypes of apolipoprotein C2 locus, creatine kinase muscle-type locus, myotonic dystrophy locus, and kallikrein 1 locus in pedigrees with PFHBI. Abridged kindred structures are shown for pedigree 2; the symbols correspond to the key in Fig 1+. The haplotypes were constructed from the results of the combination of alleles inherited at each locus in the order of the genotypes listed above (*APOCII*, *CKMM*, *DM*, and *KLK1*, respectively). Genotypes indicated by a slash indicate two indistinguishable alternatives, and haplotypes in brackets could not be assigned with certainty because of pedigree position. - indicates not tested.

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To refine the chromosomal position of the recombination event and facilitate molecular diagnosis, haplotypes for the four marker loci genotyped were deduced (Fig 3+). The haplotype of affected individuals at the *CKMM*, *DM*, and *KLK1* combined locus was 4:5:7, with the exception of a subset of pedigree 2, VII.36 and children (2:5:7), and individual IX.25, who displayed a 4:14:7 haplotype inherited from his phase-known affected father. As discussed above, a recombination event appeared to have occurred between *DM* and *KLK1* in IX.25, suggesting that the *PFHBI* locus lies telomeric to the *DM* locus.

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In this study, *PFHBI*, a familial cardiac bundle-branch conduction disorder, was genetically mapped to a group of four linked loci on chromosome 19q13.2-13.3. Multipoint mapping has positioned the gene to within a 10 cM distance, centering on the *KLK1* locus. In the families investigated, markers may now be used to support ECG-based diagnosis. This is important to the identification of persons in whom routine clinical follow-up would be advisable because of the progressive nature of the disease.<sup>1 2 3 4</sup> Furthermore, fine mapping and attempts to identify the causative gene by positional cloning can be initiated. In pedigree 2, unaffected siblings VIII.59 and VIII.60 and their affected sister VIII.58 all carry the 4:5:7 (*CKMM:DM:KLK1*) haplotype inherited from their affected father, who is homozygous at these loci. Additionally, detection of frequent recombination events between *APOC2* and rarer events between *CKMM* and *DM* and the disease currently precludes unequivocal identification of the disease-causing chromosomal region in all branches of the pedigree. Fine mapping with other markers across this region should allow identification of an invariant disease haplotype, resolving molecular diagnosis and confirming the presence of a founder effect.

A survey of known genes in the defined area focused attention on the intriguing proximity of the *PFHBI* locus to the one encoding myotonic protein kinase, which is implicated in causing myotonic dystrophy.<sup>11 12 13</sup> *PFHBI* and myotonic dystrophy have similar cardiac complications, namely bundle-branch blocks or intraventricular conduction disturbances.<sup>30 31 32 33</sup> Myotonic dystrophy is caused by instability of a CTG triplet repeat in the 3' untranslated region of the *DMK* gene,<sup>11 12 13 34 35</sup> with affected individuals having copy numbers ranging from 50 to more than 2000 repeats. The

recombination event between PFHBI and the *DM* locus and the absence of a pathologically expanded CTG repeat in PFHBI-affected individuals effectively excluded direct involvement of the *DMK* gene in PFHBI. However, it has been proposed that it is through its effects on chromosome structure that triplet repeat expansion within the *DMK* gene interferes with the expression of multiple neighboring genes, resulting in the highly variable clinical presentation that is a hallmark of myotonic dystrophy.<sup>34 36 37</sup> It can be speculated that one of these adjacent genes is involved in the specific cardiac conduction disturbances characteristic of myotonic dystrophy, and that when this gene harbors a particular mutation it causes the clinical features of PFHBI. Consequently, identifying the exact relationship of the PFHBI locus to the *DMK* locus may help elucidate the mechanisms of both diseases. Additional family members not previously available will be genotyped for possible other recombination events in the targeted region.

The exclusion of the *DMK* gene as a direct cause of PFHBI allows consideration of other candidate genes in the defined area. The highest LOD score obtained in the study, and one with no recombination events, was at the *KLK1* locus, which forms part of a linked group of kallikreins.<sup>19</sup> The kallikreins, generally considered important intravascularly, have been shown to be present in cardiac tissue in the rat.<sup>38</sup> Some of the cardiac effects of the tissue renin-angiotensin system may be mediated through a kallikrein<sup>38</sup> and could be involved in the pathophysiology of PFHBI. Included in the kallikrein gene cluster is the *R-ras* gene,<sup>39</sup> a member of the *ras* gene superfamily.<sup>40</sup> The description of a possible association of the *H-ras* proto-oncogene with the long QT syndrome,<sup>41</sup> which is characterized by cardiac arrhythmias, justifies consideration of *R-ras* as a candidate for involvement in PFHBI. A plausible mechanism may be through a role in abnormal growth or differentiation of the conduction system, a possibility proposed by Brink and Torrington in their initial description of PFHBI.<sup>1</sup>

In general, the chromosome 19q13.2-13.3 region seems particularly rich in genes with known cardiac functions or associations with cardiac pathology. The histidine-rich calcium-binding protein<sup>42</sup> is a luminal sarcoplasmic reticulum protein. It is thought that sarcoplasmic reticulum proteins may play a role in binding calcium; mutations in this gene could therefore affect intracellular calcium homeostasis. The apolipoproteins, by virtue of their connection with atherosclerosis, are often associated with cardiac dysfunction.<sup>43</sup> However, multiple recombination events at the *APOC2* locus effectively excluded not only this gene as a candidate but apolipoprotein genes *C1* and *E* as well, because all three genes are clustered within a 50-kilobase region of chromosome 19q13.2-13.3.<sup>44</sup> Similarly, the candidature of creatine kinase muscle-type, the enzyme product that plays a key role in cellular energy metabolism,<sup>45</sup> was excluded by the presence of one recombination event. The gene for troponin T also resides in the candidate region.<sup>46</sup> The troponin complex plays an important role in linking excitation to contraction of the sarcomere.<sup>47</sup> Its role in conduction tissue is uncertain, but it is known that conduction tissue is modified muscle and that it possesses rudimentary sarcomeric structures. Several other genes of unknown function<sup>37 48</sup> and HTF (*Hpa*II tiny fragments) islands,<sup>49</sup> generally associated with the 5' end of expressed genes,<sup>50</sup> have been identified in this extensively studied and gene-rich area of chromosome 19.

These results not only allow selective follow-up of individuals at risk for PFHBI, on the basis of genotypic analysis in the families studied, but define the chromosomal location of a gene causing cardiac pacing abnormalities. Additionally, establishment of the position of the loci for PFHBI and myotonic dystrophy relative to each other may shed light on the pathophysiology of both diseases. Eventually, finding and characterizing the gene that causes PFHBI may help unravel the underlying molecular mechanisms of the cardiac conduction system.

## ► Footnotes

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